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(54) Title: METHOD FOR TREATING MACROPHAGE PATHOGEN INFECTIONS

(57) Abstract

There is disclosed a method of treating an individual infected with a macrophage pathogen comprising administering an effective amount of a TGF-β antagonist. Macrophage pathogens include any pathogenic microorganism that replicates within macrophage cell hosts as their exclusive or primary host cells. TGF-β antagonists include blocking antibodies specific for a human TGF-β, soluble TGF-β receptors, protease inhibitors that inactivate a protease responsible for activating a precursor TGF-β into an active, mature TGF-β, and combinations thereof.

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TITLE

METHOD FOR TREATING MACROPHAGE PATHOGEN INFECTIONS

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a method of treating an individual infected with a macrophage pathogen comprising administering an effective amount of a Transforming Growth Factor-ß (TGF-ß) antagonist.

BACKGROUND OF THE INVENTION

Macrophage pathogens include any microorganism that replicates or proliferates within host macrophage cells as their exclusive or primary host cells. One such macrophage pathogen is *Leishmania. Leishmania* is an obligate intracellular macrophage parasite. A variety of species of *Leishmania* infect man, and most or all of such species can cause experimental infection but do not always cause disease in a predictive model such as mice. Most *L. brasiliensis* strains cause infection in mice but not disease symptoms.

Leishmania are intracellular protozoan parasites of macrophages which cause a variety of human diseases, characterized by visceral, cutaneous, or mucosal lesions. *Leishmania* are transmitted to humans and other mammals by the bite of a sandfly. Different species and isolates of *Leishmania* vary in their ability to infect and replicate in macrophages both *in vivo* and *in vitro*. For example, *L. major* and *L. amazonensis* multiply rapidly in human and mouse macrophages *in vitro*, and readily infect mice. *L. brasiliensis* is mildly to non-infectious for cultivated macrophages or mice. There have been no clear associations made between inter-species or inter-isolate properties and virulence, although development stage of the parasite is important for infectivity.

Clinically, infections with *L. brasiliensis* present as single or multiple cutaneous lesions, with a small percentage progressing to a more severe mucosal disease. While the cutaneous lesions may heal spontaneously or respond well to chemotherapy, mucosal lesions are often highly destructive and relatively refractory to treatment. Even if the mucosal lesion cures, there is often spontaneous relapse, perhaps years later. Largely due to difficulties in establishing a mouse model, there have been relatively few studies regarding immunoregulatory aspects of *L. brasiliensis* infections.

The hemoflagellate protozoan *Trypanosoma cruzi* (*T. cruzi*) causes Chagas' disease, a major public health problem in many countries of Latin America. Infection with this parasite may be acute or chronic, and frequently involves development of progressive pathology in tissues of the heart, esophagus and colon. The parasites infect a variety of nucleated cells, including macrophages. In both human and laboratory animals, T. *cruzi* infection is accompanied by a non-specific immune-suppression mediated by T cells and macrophages, which results in reduced T cell function, including helper T cells for antibody production and cytotoxicity. Mechanisms which control parasite replication during the acute and chronic phases and which maintain low but persistent numbers of circulating parasites during the chronic phase are not well understood. However, several publications point to a key role played by cytokines for regulating both parasite replication and immune responses in infected individuals.

One cytokine which is associated with both immune regulation and control of macrophage activation is Transforming Growth Factor-ß (TGF-ß). This 24 kD protein is produced by many cells, including B cells, T cells and activated macrophages. TGF-ß has been implicated as a mediator or immunosuppression, as inhibiting Interleukin-2 (IL-2) receptor induction, as mediating Interleukin-1 (IL-1) induced thymocyte proliferation, and other activities. In addition, TGF-ß has the ability to inhibit cytokine-induced macrophage activation, and to suppress production of reactive oxygen and nitrogen intermediates. The potent effects of TGF-ß on cellular immune responses often resemble those accompanying acute *T. cruzi* infection.

SUMMARY OF THE INVENTION

The present invention relates to a method of treating an individual infected with a macrophage pathogen, comprising administering an effective amount of a TGF-ß antagonist. TGF-ß antagonists comprise any pharmaceutical composition capable of blocking activity of the cytokine TGF-ß. Macrophage pathogens comprise microorganisms that replicate within macrophages as their exclusive or primary host cells. Such pathogens include, for example, *Leishmania*, *Listeria*, *Mycobacteria*, *Salmonella*, *T. cruzi*, *Pneumocystis*, and *Toxoplasma*. TGF-ß antagonists include, for example, blocking antibodies specific for a human TGF-ß, soluble TGF-ß receptors, membrane-bound TGF-ß receptors, protease inhibitors that inactivate a protease responsible for activating a precursor TGF-ß into an active, mature TGF-ß, antibodies specific to TGF-β receptors (Types I, II or III) and which prevent TGF-β binding to the receptor, and combinations thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a comparison of TGF-ß production *in vitro* induced by *Leishmania*. Active TGF-ß was produced by murine macrophages upon infection with *L. amazonensis*. Data represent the mean plus standard error of the mean of the peak response (obtained 72 hours post-infection) of three different isolates of *L. amazonensis*, compared to uninfected cells. Details of this experiment are presented in Example 1 herein.

Figure 2 shows an *in vivo* effect of TGF-ß on the course of cutaneous *Leishmaniasis*. In panel A, C57BL/6 mice were infected with *L. amazonensis*. TGF-ß was injected at 24 hours and at 6, 7, 8 and 9 weeks post infection (open circles). Control animals were injected similarly and at the same time periods with the same amount of saline (close circles). In panel B, BALB/c mice were infected with *L. brasiliensis* and recombinant TGF-ß was injected subcutaneously into the lesion at 24 hours and at weeks 1, 2, 3, 5, 12, and 13 post infection (open circles). Saline treated controls are shown with closed circles.

Figure 3 illustrates a protective effect caused by an anti-TGF-ß monoclonal antibody as a TGF-ß antagonist on the course of cutaneous *Leishmaniasis*. Panel A describes BALB/c mice infected with *L. amazonensis* and treated six times (three times per week during the first two weeks post-infection) with 1D11.16 (anti-murine TGF-ß monoclonal antibody, open circles) or a similar amount of an irrelevant antibody (closed circles). Both antibodies were injected subcutaneously at the lesion site at a volume of 30 µl. Panel B shows data obtained with BALB/c mice infected with *L. amazonensis*. During the first three week post-infection period, mice were treated three times per week with 80 µg/per dose of 1D11.16 or control saline as in Panel A.

Figure 4 is a graph showing production of active TGF-ß by *Leishmania*-infected macrophages. Cultures of mouse peritoneal macrophages were infected with *L. brasiliensis* (BA-92 or BA-331) and supernatants were collected three days later. Data are shown as the mean plus standard error of the mean from triplicate cultures.

Figure 5 shows a comparison of TGF-ß or a TGF-ß antagonist (monoclonal antibody 1D11.16) on *in vitro* replication of *Leishmania* in mouse peritoneal macrophages infected with BA-92 and cultured with 2 ng/ml TGF-ß (open bars), 200 µg/ml of 1D11.16 (hatched bars) 24 hours before and continuously after infection, or in medium alone (control bars). The numbers of intracellular parasites were determined at 2, 24, and 48 hours after infection.

Figure 6 shows the effects of exogenous TGF-ß on the course of *L. brasiliensis* infections in BALB/c mice. Infections were made with BA-92 (5×10^6 stationary-phase promastigotes) diluted in control saline or in saline containing TGF-ß at 40

μg/ml, for a total dose of 1μg/mouse. Treatments were followed with TGF-ß (1 μg/dose, open circles) or saline (closed circles) injected into the infected footpad one time per week for ten weeks.

Figure 7 shows an experiment designed similarly to Figure 6 except the BALB/c mice were infected with BA-331 instead of BA-92 in Figure 6.

Figure 8 shows the effect of TGF-ß on a previous infection with *L. brasiliensis*. BALB/c mice were infected with either BA-92 (close circles) or BA-331 (open circles) and maintained without treatment for 15 weeks. The mice were then treated with TGF-ß (1 μg/injection) or saline for a three week period for three injections per week. Saline-injected controlled mice (triangles) did not develop lesions.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of treating an individual infected with a macrophage pathogen comprising administering an effective amount of a TGF-ß antagonist. The present invention was conceived by the research finding that mice infected with the macrophage pathogen *Leishmania* can be treated with a blocking monoclonal antibody specific to murine TGF-ß. This finding, in a predictive animal model, provides data to fully enable the inventive method of treating individuals infected with any macrophage pathogen with a pharmaceutical composition comprising a substance with TGF-ß antagonist activity. Examples of TGF-ß antagonists include antibodies or monoclonal antibodies that are blocking for human TGF-ß, soluble TGF-ß receptors, membrane-bound TGF-ß receptors, protease inhibitors that inactivate a protease responsible for activating precursor TGF-ß into an active, mature TGF-ß form, and combinations thereof.

The course of infection with the protozoa and macrophage pathogen *Leishmania* is determined, in part, by their early replication in macrophages, the exclusive host cells for these organisms. Although factors contributing to the inhibition or proliferation of *Leishmania* are not well understood, certain cytokines can influence the course of infection. We have found that *Leishmania* infection induces production of active TGF-ß both *in vivo* and *in vitro*, and that TGF-ß plays an essential role in determining *in vivo* resistance and susceptibility to *Leishmania* infection in an experimental mouse model.

TGF-ß is a 24 kD protein produced by many cells, including B and T lymphocytes and activated macrophages, as well as by many other cell types. TGF-ß is generally secreted as latent precursor, requiring enzymatic cleavage of carbohydrate groups or transient acidification to release the active cytokine. Among the effects of TGF-ß on the immune system are inhibitions of IL-2-receptor induction, IL-1-induced

thymocyte proliferation and blocking of gamma interferon-induced macrophage activation.

I first investigated whether *Leishmania* infection may influence the production of TGF-ß, and thus potentially use this mechanism to survive and proliferate in macrophages. Using a sensitive bioassay, I found (*in vitro*) that infection of normal mouse peritoneal macrophages with *L. amazonensis* led to production of biologically active TGF-ß 72 hours following infection. These data are shown in Figure 1. The study was conducted by obtaining peritoneal macrophages from normal BALB/c mice after thioglycolate stimulation. The macrophages were plated at a concentration of 10^6 cells/well in a 24-well plate. Non-adherent cells were washed off the plate. The remaining adherent cells were infected with 3 x 10^6 stationary-phase *L. amazonensis* promastigotes per well for three hours. Extracellular parasites were removed and cells were cultured for another 72 hours in a humid atmosphere of 5% CO_2 at 37° C. TGF-ß was assayed using a CCL64 cell line technique as described in Silva et al., *J. Exp. Med. 174*:539, 1991.

There is increased active TGF-ß in supernatants of *Leishmania* infected macrophages. Although latent TGF-ß may be produced by uninfected macrophages, the finding of increased levels of TGF-ß in culture supernatants of infected macrophages appears to indicate one mechanism by which *Leishmania* evade host defense by macrophages. Therefore, these data provide an important understanding regarding pathogenesis of infection of macrophage pathogens.

The potential role of TGF-ß during infection, *in vivo*, of a macrophage pathogen was investigated. *Leishmania* lesions were examined using a murine TGF-ß-specific monoclonal antibody. There was local TGF-ß production in mouse footpads following *Leishmania* infection but there was no detectable TGF-ß in uninfected footpads of the same mouse. Therefore, increased TGF-ß production was associated with *Leishmania* infection both *in vitro* and *in vivo*.

Leishmania resistant C57BL/6 mice will normally heal after *Leishmania* infections. When such mice were infected with *Leishmania*, and treated with recombinant TGF-ß, the TGF-ß treated mice developed large, non-healing lesions (Figure 2). These data indicate that TGF-ß is able to reverse genetic resistance to *Leishmaniasis* in C57BL/6 mice.

TGF-ß also influences infection with *L. brasiliensis*. This parasite does not produce lesions even in BALB/c mice, which are susceptible to infection with other *Leishmania* species. However, TGF-ß treatment led to replication of *L. brasiliensis* and to production of lesions in BALB/c mice (Figure 2B). When the lesions in the BALB/c mice were investigated histologically, lesions from saline-treated animals were

composed of predominately lymphocytic infiltrate, whereas lesions from TGF-ß-treated BALB/c mice exhibited a large number of heavily vacuolated and parasitized macrophages among the lymphocytes. Therefore, local administration of TGF-ß led to production of acute disease in two different animal models of resistance.

In view of the finding that TGF-ß was increased during *Leishmania* infection and that exogenously-administered recombinant TGF-ß could produce exacerbated infections in resistant mice, I investigated whether endogenously-produced TGF-ß influenced *Leishmania* infection *in vivo*. Highly susceptible BALB/c mice were treated with a neutralizing antibody specific for murine TGF-ß during the course of infection with *L. amazonensis*. Control mice were similarly injected with an irrelevant immunoglobulin. *In vivo* treatment with anti-TGF-ß arrested development of lesions resulting from *L. amazonensis* infection in susceptible BALB/c mice (Figure 3). Inhibition of lesion development was noted by the 5th week post-infection and was maintained thereafter. Therefore, endogenous TGF-ß production was directly associated with susceptibility to a macrophage pathogen such as *Leishmania*. Moreover, anti-TGF-ß antibody is a TGF-ß antagonist that will have similar biological and pharmacological properties to other TGF-ß antagonists, such as other blocking monoclonal antibodies specific for a human TGF-ß, soluble TGF-ß receptors that bind and inactivate active TGF-ß, and protease inhibitors that inactivate a protease responsible for activating a precursor TGF-ß into an active, mature TGF-ß.

Production of active TGF-ß requires infection by live macrophage pathogens or other parasites, and represents an important parasite virulence mechanism. In addition, exogenously-administered TGF-ß was shown to have profound effects on the course of macrophage parasite infection. Genetically resistant mice became susceptible to macrophage parasite infection following TGF-ß treatment, and avirulent *Leishmania* produced disease in conjunction with TGF-ß. In the aggregate, these data demonstrate a unique role for TGF-ß in mediating resistance and susceptibility to a parasitic protozoan, and represent an important mechanism by which macrophage pathogens avoid destruction by host macrophages. The data further predict and enable the full scope of the claimed invention.

Example 1

This example illustrates that active TGF-ß is produced *in vitro* by macrophages infected with *Leishmania*. Macrophages are both host cells for *Leishmania* and other macrophage parasites and important producers of the cytokine TGF-ß. I analyzed the production of TGF-ß during *in vitro* infection of peritoneal macrophages from normal mice. Thioglycolate-stimulated mouse peritoneal macrophages were plated in 24-well

plates at 5 x 10^5 cells/well in RPMI1640 supplemented with antibiotics, glutamine, and 10% heat-inactivated fetal calf serum. After three days in culture, macrophages were infected with stationary-phase *L. brasiliensis* promastigotes (2.5 x 10^6 parasites/well) for three hours. Extracellular parasites were removed by extensive washing with wash culture medium, followed by further incubation for up to 72 hours. Cell-free supernatants were collected at 24, 48, or 72 hours after infection. Replicate monolayers were washed with phosphate buffered saline (PBS), fixed in acetone-methanol, and stained with Giemsa. The percentage of infected macrophages, and the number of amastigotes per 100 macrophages were determined by microscopic examination as described in Reed et al., *J. Exp. Med. 166*:1734, 1987.

TGF-ß was assayed for presence and activity by taking supernatants from uninfected and *Leishmania*-infected macrophage cultures and assaying for TGF-ß activity using CCL-64 mink lung epithelial cells as described in Ranchalis et al., *Biochem. Biophys. Res. Commun. 148*:783, 1987. Cells were plated at 10^5 cells/well into 96-well microtiter plates for one hour at 37° C in volume of 100 µl of DMEM (Dulbecco's Minimal Essential Media) containing 1% heat-inactivated fetal calf serum. After one hour, TGF-ß standards (ranging in concentration from 2 ng to 0.00049 ng) or test supernatants were added at appropriate dilutions to a final volume of 200 µl/well. The standards were diluted by 2x serial dilutions in DMEM containing 10% heat-inactivated fetal calf serum. The cultures were incubated for 22 hours at 37° C and tritiated (^{3}H) thymidine was added during the final four hours at a concentration of 0.25 µCi/well. The plates were placed in a -70° C freezer for at least one hour, thawed, and harvested onto glass filter fibers. Radioactivity was determined by liquid scintillation counting.

TGF-ß released in its latent or precursor form will not inhibit CCL-64 cell cultures (Twardzik et al., in *Transforming Growth Factors-Chemistry, Biology, and Therapeutics*, Ann. N.Y. Acad. Sci. *593*:276, 1990). Hydrochloric acid (1N) was added to each culture supernatant to adjust the pH to 3.0-3.5 for 10 minutes at room temperature and immediately neutralized to pH 7.0-7.6 by adding 1N NaOH. The concentration of TGF-ß in each sample was determined by comparison with a curve generated from the TGF-ß standards. A sample regression curve was computed using a Microsoft GB STAT® program.

The production of TGF-ß in the supernatant of infected macrophages was determined at several time points after infection. Two interesting observations came from these experiments. The first was that biologically active TGF-ß was produced by mouse peritoneal macrophages following *in vitro* infection with *Leishmania* (Figure 4).

The second was that the levels of TGF-ß induced differed between two individual isolates of *L. brasiliensis* (BA-92 and BA-331).

Example 2

This example illustrates the effect of TGF-ß to exacerbate *in vitro* infection by *L. brasiliensis*. *In vitro* infection of mouse macrophages by *L. brasiliensis* is characterized by static numbers of intracellular parasites. However, when macrophages were treated with recombinant TGF-ß prior to infection, a progressive increase in numbers of intracellular amastigotes occurred (Figure 5). Therefore, explanted macrophages were capable of limiting the intracellular replication of *Leishmania*, and the mechanism responsible for this control was inhibited by exogenous TGF-ß. Regulation of intracellular parasite replication was resisted due to progressive reduction of amastigotes in macrophages treated with a neutralizing monoclonal antibody (mAb) specific for TGF-ß. This observation suggests a role for *Leishmania*-induced TGF-ß in the persistence of intracellular macrophage parasites.

Example 3

This example illustrates the exacerbation of *in vivo Leishmania* infection by TGF-ß. In examples 1 and 2, *in vitro* observations suggested a differential ability of *Leishmania* isolates to induce active TGF-ß production as well as a role for TGF-ß in control of *Leishmania* infection. This example illustrates *in vivo* experiments in which two *L. brasiliensis* isolates were used to infect mice with and without administering TGF-ß.

BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were infected in the left hind footpad with 5×10^6 stationary-phase promastigotes of *Leishmania* in 25 µl of saline. The parasites were resuspended in saline containing 40 µg/ml rTGF-ß (1 µg/mouse). Recombinant TGF-ß treatments were at doses of 1 µg/mouse injected into each infected footpad. Control mice were injected with the same volume of saline. Lesion progression was evaluated during the course of infection by measuring footpad thickness with a dial gauge caliper (C. Starret, Athol, MA) and expressed as lesion size in mm (infected footpad thickness minus uninfected contralateral footpad thickness). At different periods of time after infection, animals were sacrificed, infected footpads were removed, fixed in 10% buffered formalin, processed and stained with hematoxylin and eosin.

Each isolate of *L. brasiliensis* produced characteristic self-limited infections in saline-treated mice (Figures 6 and 7). There was a direct correlation seen between lesion size and TGF-ß levels induced by the two parasite isolates. Thus, BA-331 was a

relatively weak inducer of TGF-ß *in vitro* and produced no measurable lesion, while BA-92 induced measurable but self-limiting lesions as well as significantly higher *in vitro* levels of TGF-ß. These data suggest a role for TGF-ß production in *Leishmania* infectivity and/or intracellular replication *in vivo*.

Exogenous TGF-ß was administered to groups of mice infected with either of these two *L. brasiliensis* isolates. Recombinant TGF-ß treatment led to a dramatic increase in mean lesion size in all mice (Figures 6 and 7). Histologically, the lesions were characterized by large numbers of heavily parasitized macrophages. The differences between recombinant TGF-ß-treated and saline-treated mice were greatest in those mice infected with BA-331, which produced measurable lesions only in the presence of TGF-ß (Figure 6). By inducing or exacerbating lesions in *L. brasiliensis* infected mice, the role for TGF-ß in the pathogenesis of *Leishmaniasis* was established.

Example 4

This example illustrates activation of latent *L. brasiliensis* infections by administration of exogenous TGF-ß. *L. brasiliensis* infection in intact mice is characterized by transient or no lesion development as illustrated in Figure 6. To determine whether parasites persisted following infection and whether recombinant TGF-ß could influence prior infections, BALB/c mice were treated with recombinant TGF-ß for a three week period, beginning 15 weeks after initial infection. When recombinant TGF-ß was administered there were no visible lesions present characteristic of disease. Animals were treated with recombinant TGF-ß (1µg/mice/dose) or saline, three times a week during a three week period. In both groups, injection of recombinant TGF-ß but not saline, led to lesion development that was detected two to three weeks after initiation of treatments (Figure 8). Even though TGF-ß treatment was continued for only three weeks, progressive lesion growth continued throughout a 15 week observation period. Saline treated mice exhibited no change in lesion size. These data illustrate that persistent latency of *L. brasiliensis* infection in mice and further demonstrate the ability of TGF-ß to mediate disease resistance and susceptibility.

I claim:

1. A method of treating an individual infected with a macrophage pathogen comprising administering an effective amount of a TGF-ß antagonist.

2. The method of claim 1 wherein the TGF-ß antagonist is selected from the group consisting of blocking monoclonal antibodies specific for a human TGF-ß, protease inhibitors that inactivate a protease responsible for activating a precursor TGF-ß into an active, mature TGF-ß, soluble TGF-ß receptors, and combinations thereof.

3. The method of claim 1 wherein the macrophage pathogen is a pathogenic microorganism that replicates within macrophage cell hosts as their exclusive or primary host cells.

4. The method of claim 3 wherein the macrophage pathogen is selected from the group consisting of *T. cruzi, Salmonella, Pneumocystis, Toxoplasma, Listeria, Tuberculosis* and *Leishmania.*

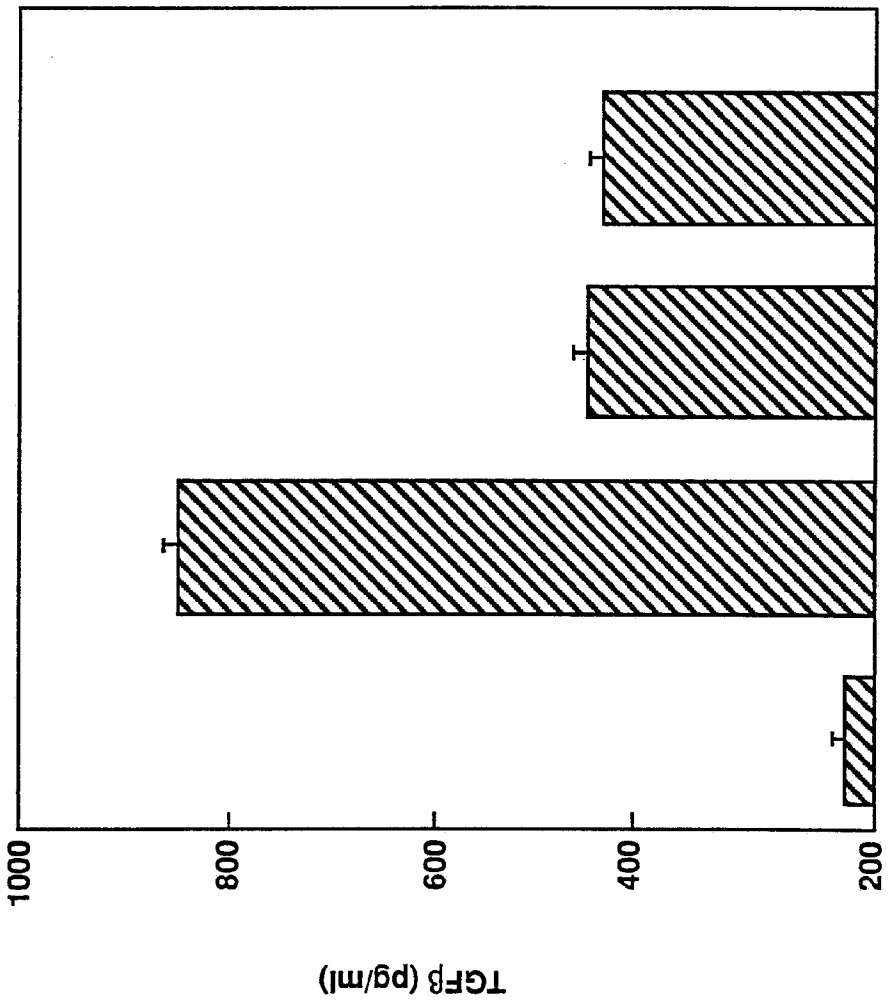

Figure 1

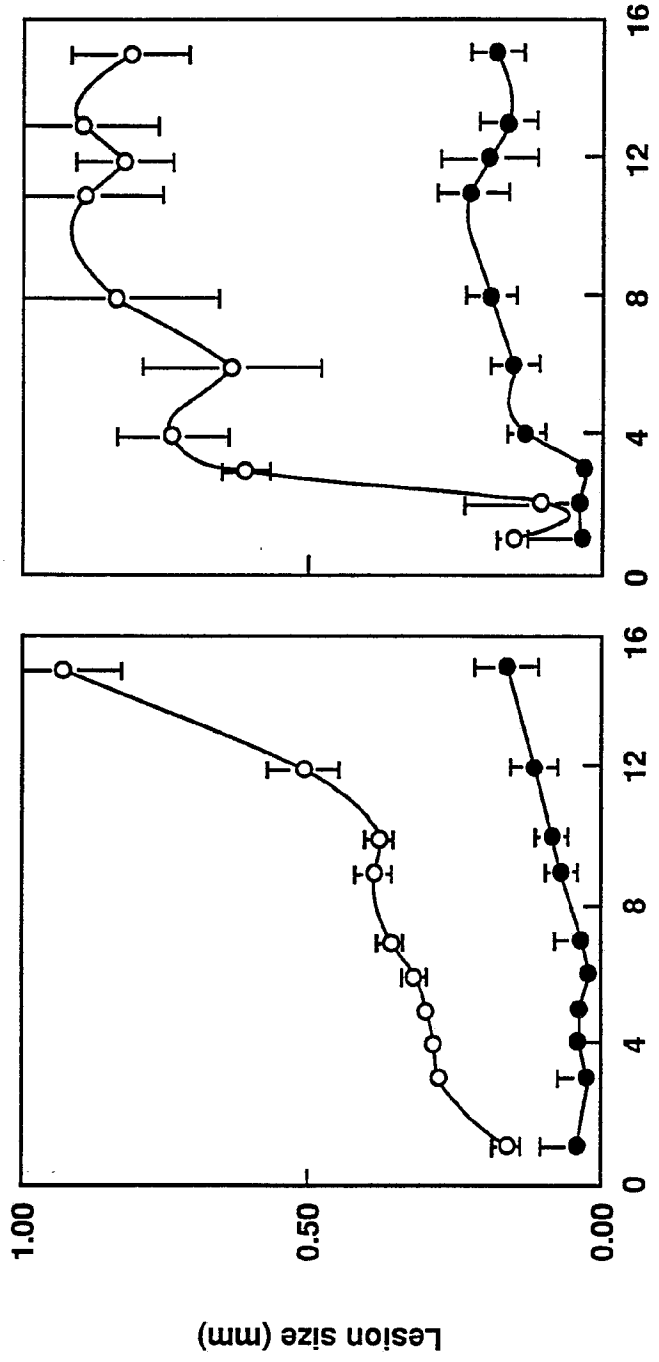

Figure 2A

Figure 2B

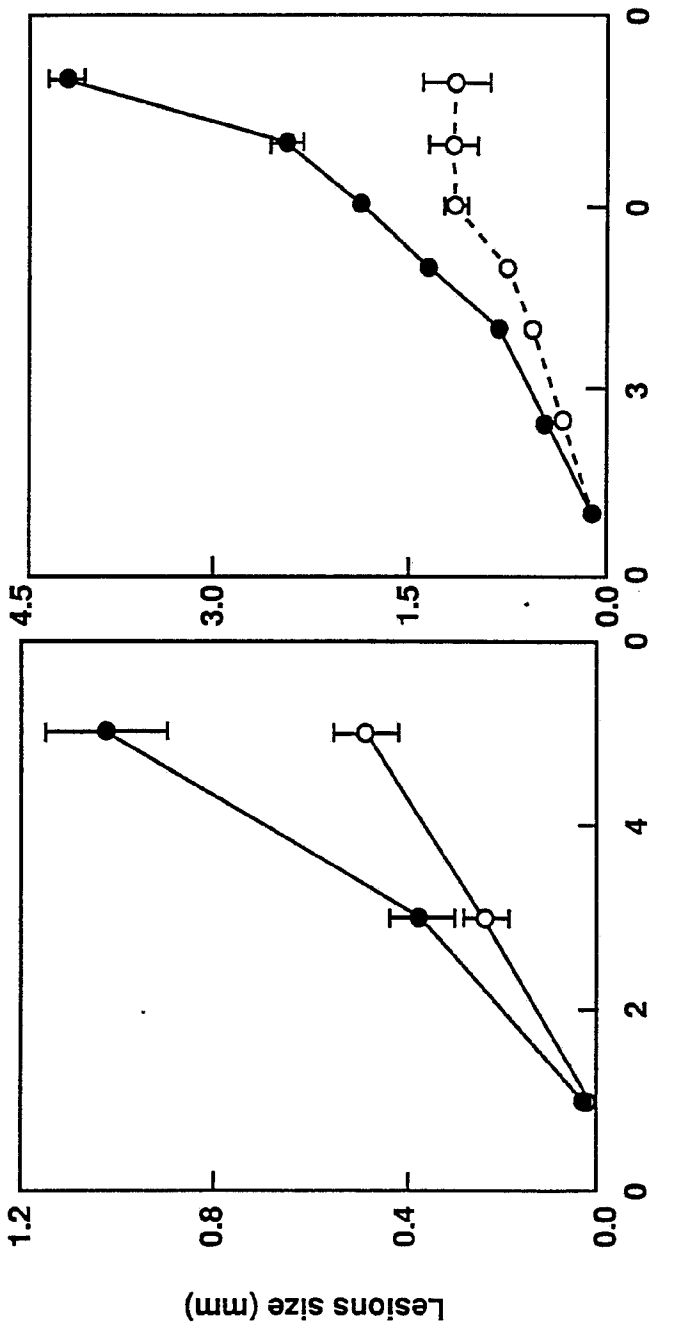

Figure 3A

Figure 3B

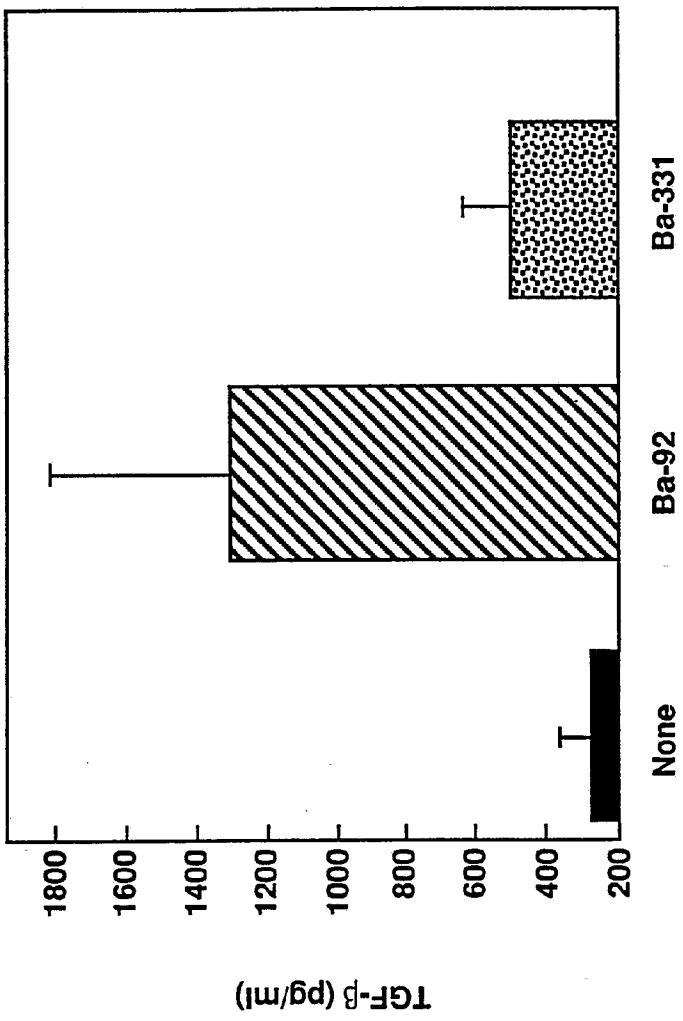
TGF-β (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
None
Ba-92
Ba-331

Figure 4

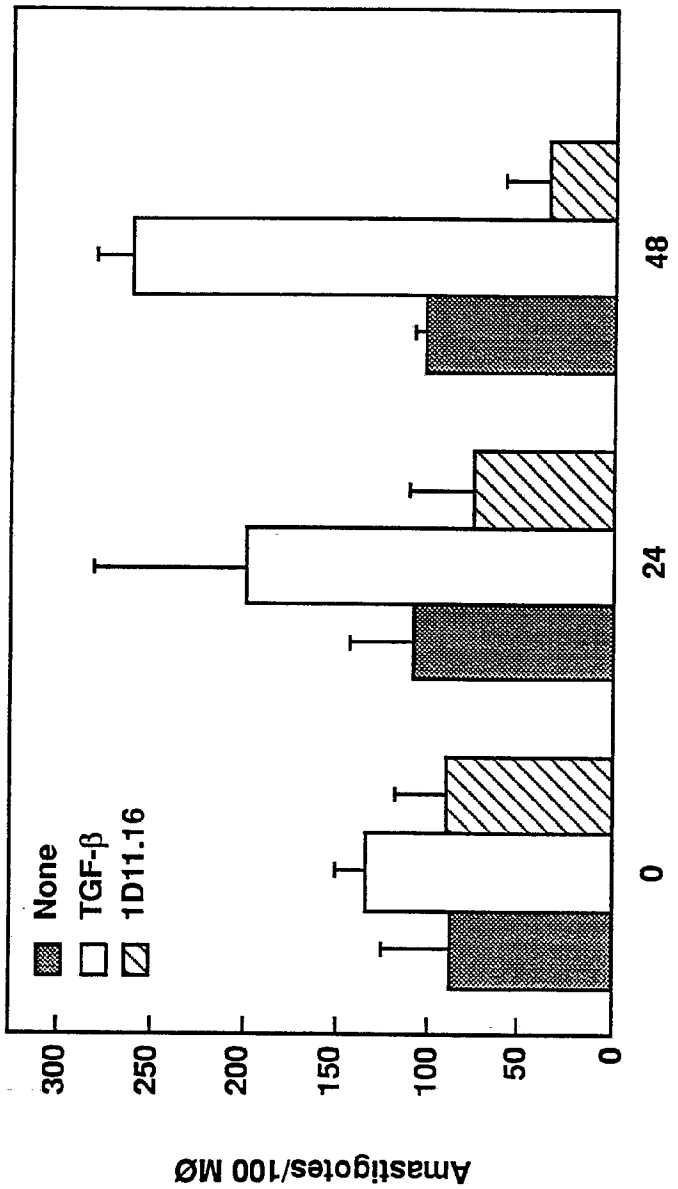
Amastigotes/100 MØ
300
250
200
150
100
50
0
None
TGF-β
1D11.16
0
24
48
Hours after Infection

Figure 5

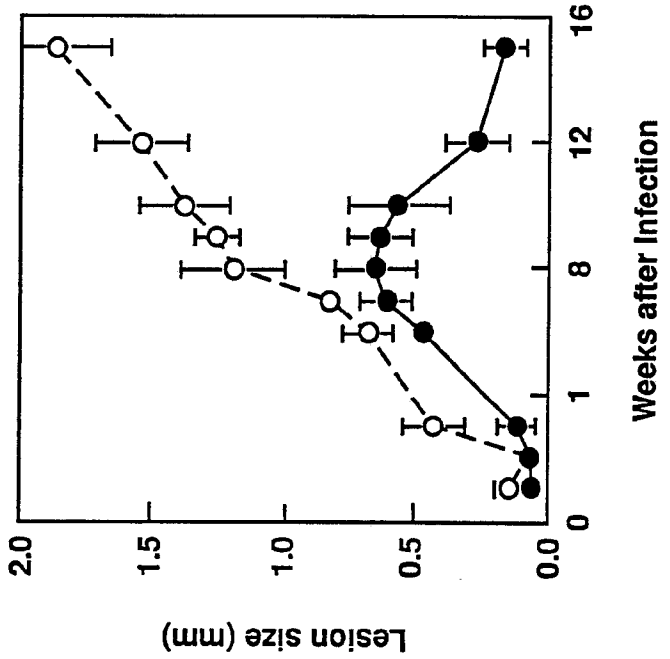

Figure 6

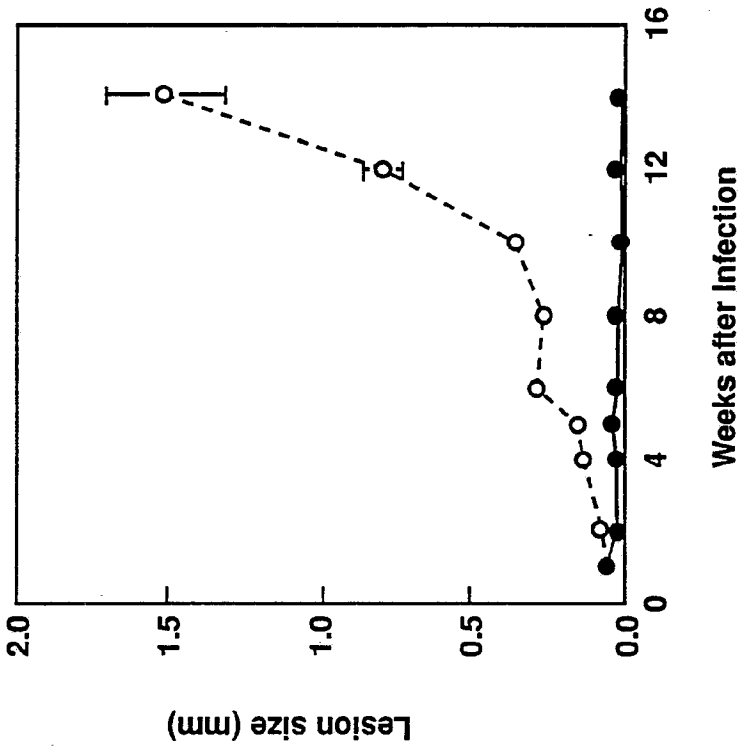

Figure 7

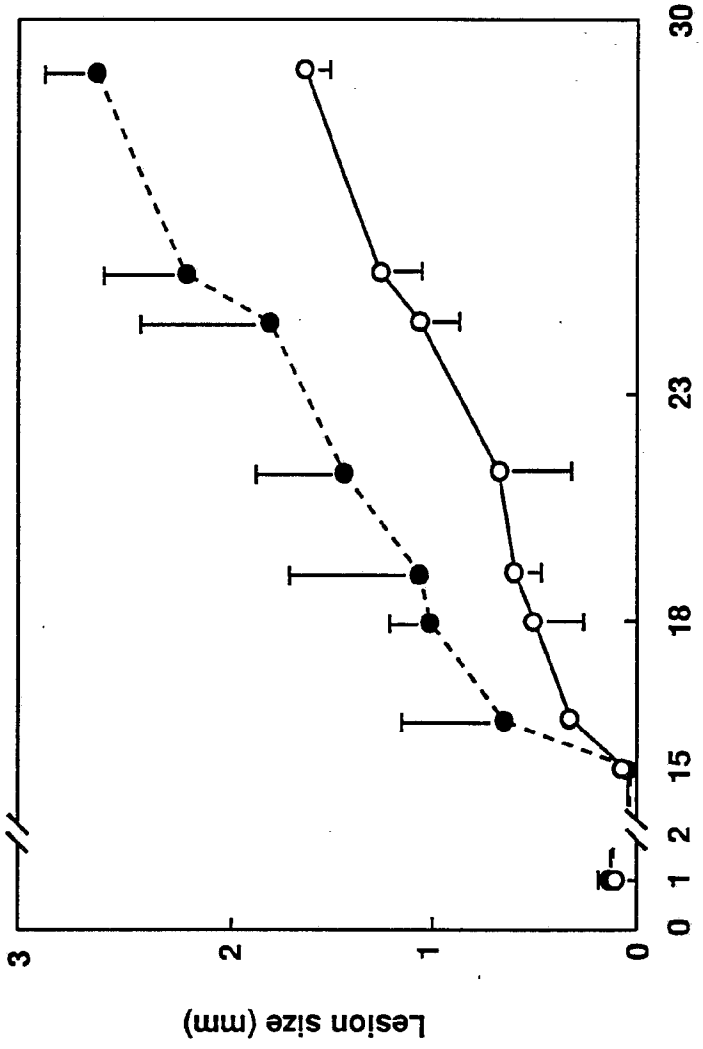

Figure 8

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02017

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/395, 39/00; C07K 15/28
US CL :424/85.8, 88; 530/387.1, 388.23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8, 88; 530/387.1, 388.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	Science, Volume 257, Number 5069, issued 24 July 1992, Barral-Netto et. al. "Transforming Growth Factor Beta, in leishmanial infection: a parasite escape mechanism", pages 545-548, see entire article.	1-4
Y	Journal of Experimental Medicine, Volume 174, Number 3, issued 01 September 1991, Silva et. al., "Regulation of Trypanisoma cruzi infections in vitro and in vivo by transforming growth factor beta", pages 539-545, see entire article.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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